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(54) Title: IMMOBILIZED REAGENTS FOR KINASE ASSAYS			
<p><b>SCINTI-PI(4,5)P<sub>2</sub></b></p>			
(57) Abstract			
<p>There is disclosed a composition represented by the formula <math>\text{PIP}_n\text{-L-S-Matrix}</math>, wherein <math>\text{PIP}_n</math> is a phosphoinositide polyphosphate, L is a linker moiety, S is a scintillant, and Matrix is a solid support. Preferred linker moieties include succinimide and poly(ethylene glycol) linkers, and a preferred scintillant is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole. In a preferred embodiment, the Matrix is a microtitre plate. The composition can be used for assaying phosphatidylinositol kinases and for screening compounds for inhibition of phosphatidylinositol kinase activity. Methods of making and using the compound are also disclosed.</p>			

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## IMMOBILIZED REAGENTS FOR KINASE ASSAYS

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/090,922, filed June 26, 1999, which is hereby incorporated by reference.

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STATEMENT REGARDING FEDERALLY SPONSORED  
RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant No. NS-29632 awarded by the National Institutes of Health. The government has certain rights in the invention.

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## BACKGROUND OF THE INVENTION

This invention relates to *in vitro* assays of kinase activity. More particularly, the invention relates to scintillant-tethered phosphoinositide polyphosphates (PIP<sub>n</sub>s), methods of making thereof, and methods of use thereof.

15

Phosphoinositide (PI) metabolism plays an important role in the control of diverse cellular processes such as proliferation and differentiation, apoptosis, and control of cell shape and cell migration. P. DeCamilli et al., 271 Science 1533-1539 (1996); P. Janmey, 2 Chem. & Biol. 61-65 (1995); J.E. Rothman, 5 Protein Sci. 185-194 (1996); R. Schekman & L. Orci, 271 Science 1526-1533 (1996); S. Cockcroft, 35 Prog. Lipid Res. 345-370 (1996). The regulation of cell survival is a central theme in normal embryological development as well as in diseases such as cancer, T-cell depletion associated with immune deficiency syndrome, and neural degeneration. T.F. Franke & L.C. Cantley, 390 Nature 116-117 (1997); T.J. McDonnell et al., 52 Experientia 1008-1017 (1996). Recent studies have implicated phosphoinositide 3-kinase (PI3K) in a distinct pathway that conveys survival signals from various mammalian cell-surface receptors, A. Toker & L.C. Cantley, 387 Nature 6773-676 (1997); W.G. King et al., 17 Mol. Cell. Biol. 4406-4418 (1997); T.R. Franke et al., 275 Science 665-668 (1997), including growth factors, oncoproteins, and non-mitogenic stimuli. The discovery of PI3K came from its association with several oncogene products. C.L. Carpenter et al., 13 Mol. Cell. Biol. 1657-1665 (1993). Inhibitors of PI3K, such as

wortmannin, G.D. Prestwich, 7 Chemtracts-Org. Chem. 301-305 (1994); R. Yao & G.M. Cooper, 267 Science 2003-2006 (1995), and LY294002, have further confirmed these distinct cellular functions. C.L. Carpenter & L.C. Cantley, 1288 Biochim. Biophys. Acta M11-M16 (1996). PI3K may also be involved in mediating several insulin-regulated metabolic pathways, P.R. Shepherd et al., 17 J. Mol. Endocrinol. 175-184 (1996), leading to diabetes, including glucose uptake, antilipolysis, glycogen synthesis, and the suppression of hepatic gluconeogenesis. D.J. Withers et al., 391 Nature 900-904 (1998).

The PI3K family of lipid kinases includes PI3K $\gamma$ , a G protein-activated PI3K, and the yeast PI3K encoded by the Vps34 gene. PI3Ks are responsible for the phosphorylation of inositol lipids via transfer of the  $\gamma$  phosphate of ATP to the D-3 hydroxyl position. B. Vanhaesebroeck et al., 22 TIBS 267-272 (1997); L.K. MacDougall et al., 5 Curr. Biol. 1404-1415 (1995); J. Domin & M.D. Waterfield, 410 FEBS Lett. 91-95 (1997); C.L. Carpenter & L.C. Cantley, 8 Curr. Opin. Cell Biol. 153-158 (1996). In many systems, PI3K is a heterodimeric protein consisting of an 85 kDa regulatory subunit (p85) that contains two Src homology-2 (SH2) domains and an SH3 domain, and a 110 kDa catalytic subunit (p110) in which kinase activity resides. C.L. Carpenter et al., 265 J. Biol. Chem. 19704-19711 (1990). The heterodimeric PI3-kinases and PI3K  $\gamma$  phosphorylate at the D-3 position of the inositol ring of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI(4)P), and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>), giving rise to phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P<sub>2</sub>), and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>) (FIG. 1). However, yeast Vps34p kinase has a substrate specificity restricted to PI. M. Susa et al., 267 J. Biol. Chem. 22951-22956 (1992); B. Vanhaesebroeck et al., 27 Cancer Surv. 249-270 (1996).

As various growth factors and cytokines bind to their cell-surface receptors, intracellular PI3K becomes activated. At the membrane, PI3K converts PI(4,5)P<sub>2</sub> to PI(3,4,5)P<sub>3</sub> as well as generating PI(3,4)P<sub>2</sub> from inositol 5'-polyphosphate (FIG. 2). Recently, a novel naturally occurring membrane bisphosphate, phosphatidylinositol 3,5-bisphosphate (PI(3,5)P<sub>2</sub>), J. Peng & G.D. Prestwich, Tetrahedron Lett. (1998); C.C. Whiteford et al., 323 J. Biochem. 597-601 (1997), was identified with unknown biological relevance. However, evidence is available for its production via action of a PI3K on phosphatidylinositol 5-phosphate (PI(5)P) or of a phosphoinositide 5-kinase (PI5K) on PI(3)P. The lipid products of

PI3K, PI(3,4)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub>, J.K. Klarlund et al., 275 Science 1927-1930 (1997); M.P. Derman et al., 272 J. Biol. Chem. 6465-6470 (1997); D. Stokoe et al., 277 Science 567-570 (1997), act as second messenger molecules and induce activity of multiple downstream effectors that include SH2 and pleckstrin homology (PH) domains of serine/threonine and tyrosine kinases as well as various cytoskeletal proteins. T.F. Franke et al., 275 Science 665-668 (1997); C.L. Carpenter & L.C. Cantley, 1288 Biochim. Biophys. Acta M11-M16 (1996); C.L. Carpenter et al., 265 J. Biol. Chem. 19704-19711 (1990). Activation of the serine/threonine kinase, Akt, and phosphoinositide-dependent kinases I (PDK1) are known to inhibit apoptosis (programmed cell death) by phosphorylating a critical serine residue on the 5 agonist protein BAD. A. Toker & L.C. Cantley, 387 Nature 673-676 (1997); S.R. Datta et al., 91 Cell 231-241 (1997); L. Del-Peso et al., 278 Science 687-689 (1997). BAD promotes cell 10 death by blocking the activity of the cell survival factors, Bax-Bcl-x<sub>L</sub> by displacing Bax and binding to Bcl-x<sub>L</sub>. E. Yang et al., 80 Cell 285-291 (1995). Once BAD is dissociated from Bcl-x<sub>L</sub>, it reassociates with the 14-3-3 cytosol binding site, and the Bcl-x<sub>L</sub> becomes capable of 15 promoting cell survival by repressing apoptosis pathways involving the activity of Apaf-1, cytochrome c (cyt.c), and the caspase protease cascade. T.F. Franke & L.C. Cantley, 390 Nature 116-117 (1997).

Most current kinase assays require separation of unlabeled  $\gamma$ -<sup>32</sup>P-ATP or  $\gamma$ -<sup>33</sup>P-ATP from the phosphorylated product, and then quantitation by liquid scintillation counting. Such 20 assays are laborious to carry out, difficult to automate, require addition of scintillation cocktail, and generate problems and expenses associated with radiowaste disposal.

In view of the foregoing, it will be appreciated that providing compositions and methods that would expedite the processing of samples in kinase assays and would be more amenable to automation would be significant advancements in the art. Such assays would 25 require no addition of scintillation cocktail and would be less problematic and less expensive for radiowaste disposal. It will be further appreciated that providing scintillant-tethered phosphoinositide polyphosphates and methods of making and using thereof would be significant advancements in the art.

#### BRIEF SUMMARY OF THE INVENTION

30 It is an object of the present invention to provide scintillant-tethered phosphoinositide

polyphosphates.

It is also an object of the invention to provide a method of making scintillant-tethered phosphoinositide polyphosphates.

It is another object of the invention to provide a method using scintillant-tethered phosphoinositide polyphosphates.

It is still another object of the invention to provide substrate-bound scintillant-tethered phosphoinositide polyphosphates.

It is yet another object of the invention to provide a method for assaying phosphatidylinositol kinases.

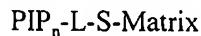
10 It is a still further object of the invention to provide a method for screening compounds for interference with phosphatidylinositol kinase activity.

These and other objects can be achieved by providing a composition represented by the formula



15 wherein  $\text{PIP}_n$  is a phosphoinositide polyphosphate, L in a linker moiety, and S is a scintillant. In a preferred embodiment,  $\text{PIP}_n$  is a member selected from the group consisting of PI, PI(3)P, PI(4)P, PI(5)P, PI(4,5)P<sub>2</sub>, PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub>. In another preferred embodiment, L is a member selected from the group consisting of succinimide and poly(ethylene glycol) linkers. In still another preferred embodiment, S is 2-(4-amino-20 methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (i.e., amino-PBD).

Another aspect of the invention comprises a composition represented by the formula



25 wherein  $\text{PIP}_n$  is a phosphoinositide polyphosphate, L in a linker moiety, S is a scintillant, and Matrix is a solid support. In a preferred embodiment,  $\text{PIP}_n$  is a member selected from the group consisting of PI, PI(3)P, PI(4)P, PI(5)P, PI(4,5)P<sub>2</sub>, PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub>. In another preferred embodiment, L is a member selected from the group consisting of succinimide and poly(ethylene glycol) linkers. In still another preferred embodiment, S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (i.e., amino-PBD). The Matrix is preferably a hydrophobic polymer and more preferably polystyrene. In an especially preferred embodiment of the invention the Matrix is in the form of a microtiter plate.

A method for assaying a phosphatidylinositol kinase comprises:

(a) providing a composition represented by the formula



wherein  $\text{PIP}_n$  is a phosphoinositide polyphosphate, L in a linker moiety, S is a scintillant, and

5 Matrix is a solid support;

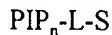
(b) adding to the composition effective amounts of a sample to be tested containing the phosphatidylinositol kinase, reaction buffer, and ATP labeled with a low-energy  $\beta$ -emitter to form a reaction mixture;

(c) incubating the reaction mixture for a sufficient time and under suitable 10 conditions for the enzyme to phosphorylate the  $\text{PIP}_n$  with the low-energy  $\beta$ -emitter, whereupon radioactive decay of the low-energy  $\beta$ -emitter induces emission of light by the scintillant; and

(d) detecting the light.

In a preferred embodiment of this method, the low-energy  $\beta$ -emitter is  $^{33}\text{P}$ .

15 A method of making a composition represented by the formula

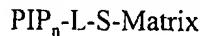


wherein  $\text{PIP}_n$  is a phosphoinositide polyphosphate, L in a linker moiety, and S is a scintillant comprises:

(a) activating the scintillant to result in an activated scintillant, and reacting the 20 activated scintillant with the linker moiety, thereby obtaining a linker-scintillant intermediate;

(b) activating the linker-scintillant to result in an activated linker-scintillant intermediate, and reacting the activated linker-scintillant intermediate with an aminoacyl- $\text{PIP}_n$ , thereby obtaining the composition represented by the formula  $\text{PIP}_n\text{-L-S}$ .

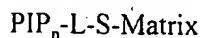
A method of making a composition represented by the formula



wherein  $\text{PIP}_n$  is a phosphoinositide polyphosphate, L in a linker moiety, S is a scintillant, and Matrix is a solid support comprises adsorbing  $\text{PIP}_n\text{-L-S}$  to the Matrix.

A method for screening compounds for a drug that interferes with phosphatidylinositol kinase activity comprises:

30 (a) providing a composition represented by the formula



wherein  $\text{PIP}_n$  is a phosphoinositide polyphosphate, L is a linker moiety, S is a scintillant, and Matrix is a solid support;

(b) contacting the composition with effective amounts of a compound to be tested, reaction buffer, phosphatidylinositol kinase, and ATP labeled with a low-energy  $\beta$ -emitter to 5 form a reaction mixture;

(c) incubating the reaction mixture for a sufficient time and under suitable conditions for the phosphatidylinositol kinase to phosphorylate the  $\text{PIP}_n$  such that the low-energy  $\beta$ -emitter is coupled thereto, whereupon radioactive decay of the low-energy  $\beta$ -emitter induces emission of light by the scintillant;

10 (d) measuring the light and comparing the amount of light measured to an amount of light emitted from a control reaction that lacks the compound, wherein a decrease of light in the presence of the compound indicates interference with the phosphatidylinositol kinase activity.

#### BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

15 FIG. 1 shows selected phosphoinositide kinase pathways.

FIG. 2 shows that phosphorylation of Akt by  $\text{PIP}_3$ -activated PDK1 blocks caspase activation and prevents apoptosis.

FIG. 3 shows a representative SCINTI-PIP and its graphic representation.

FIG. 4 shows the principle of the scintillation proximity assay (SPA).

20 FIG. 5 shows chemical structures,  $\lambda_{\text{abs}}$ , and  $\lambda_{\text{em}}$  of common scintillants.

FIG. 6 shows commercially available phosphoinositide polyphosphates.

FIG. 7 shows synthesis of *sn*-1-*O*-(6-aminohexanoyl), 2-*O*-hexanoyl- $\text{PI}(4,5)\text{P}_2$ .

FIG. 8 shows modified  $\text{PIP}_n$ s that are substrates for PI3K.

25 FIG. 9 shows chemical synthesis of 2-(4-aminomethylphenyl)-5-(*b*-biphenyl)-1,3,4-oxadiazole (amino-PBD); reagents: a,  $\text{MeOH}/\text{H}_2\text{SO}_4$ ; b,  $\text{H}_2\text{NNH}_2$ ; c, toluene/heat; d,  $\text{SOCl}_2$ /heat; e,  $\text{LiAlH}_4/\text{THF}$ .

FIG. 10 shows synthesis of three SCINTI-LINKER modules from desymmetrized PEG.

30 FIG. 11 shows coupling of SCINTI-LINKER modules to four PIP scaffolds to give SCINTI-PIPs; for PEG-6 and PEG-85, X may be either NH or O.

FIG. 12 shows the principle of scintillation proximity assay as applied to SCINTI-PIP.

#### DETAILED DESCRIPTION

Before the present compositions, method of making thereof, and method of use thereof are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out herein.

As used herein, "comprising," "including," "containing," "characterized by," and grammatical equivalents thereof are inclusive or open-ended terms that do not exclude additional, unrecited elements or method steps. "Comprising" is to be interpreted as including the more restrictive terms "consisting of" and "consisting essentially of."

As used herein, "consisting of" and grammatical equivalents thereof exclude any element, step, or ingredient not specified in the claim.

As used herein, "consisting essentially of" and grammatical equivalents thereof limit the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic or characteristics of the claimed invention.

As used herein, "effective amount" means an amount sufficient to provide a selected or desired result. For example, an effective amount of a phosphatidylinositol kinase for carrying out an assay thereof is an amount of such enzyme that will phosphorylate a selected amount of substrate in a selected period of time under standard conditions. This amount can be easily determined without undue experimentation by a person skilled in the art. Similarly, effective amounts of reaction buffer and ATP for carrying out a phosphatidylinositol kinase assay are amounts known in the art or amounts that can be readily determined by a person skilled in the art for obtaining a selected result.

PIP<sub>n</sub>s are key signaling molecules in cellular communication. PIP<sub>n</sub>s are biosynthesized by the interplay of kinases and phosphatases. In particular, isozymes of PI 3-kinase, PI 4-kinase, and PI 5-kinase have been identified and are important in insulin signaling, apoptosis, cytoskeletal remodeling, and protein trafficking. Few specific inhibitors 5 for these enzymes exist. The development of targeted therapeutic agents is substantially enhanced with rapid assays for lipid kinase activities that can be used in, for example, a 96-well format. Vast libraries of potential inhibitors can be screened with such a high throughput assay. The present assay expedites the processing of samples and can be automated. The assay requires no addition of scintillation cocktail and is less problematic 10 and less expensive for radiowaste disposal.

The present invention, in which a scintillant is covalently linked to a substrate molecule, can be used for assaying any PI kinase. A substantial market exists for high-throughput kits according to the present invention, which facilitate screening of combinatorial libraries of compounds for potential inhibitors of lipid kinases. Screening of multiple cell 15 extracts allows rapid detection of tissue- or physiology-specific activities. Screening of libraries of compounds allows detection of potential isozyme-specific inhibitors as therapeutic leads.

#### Method for Screening Inhibitors of PI3K

A method for screening inhibitors of PI3K uses immobilized PIP<sub>n</sub>s, referred to herein 20 as SCINTI-PIPS (scintillant phosphoinositide polyphosphates). SCINTI-PIPS are a class of hybrid fluorophore-phosphoinositide polyphosphates that can be used to immobilize a PIP<sub>n</sub> substrate on a plastic surface, as shown in FIG. 3. Similar to the scintillation proximity assay (SPA; Amersham) and FLASHPLATE (NEN Life Science Products) technologies, N. Nelson, 165 Anal. Biochem. 287-293 (1986); N. Bosworth & P. Towers, 341 Nature 167-168 25 (1989); S. Undenfried et al., 161 Anal. Biochem. 494-500 (1987), the present technique relies on the scintillant only producing a detectable visible photon when low energy  $\beta$ -emitters, such as <sup>3</sup>H, <sup>14</sup>C, or <sup>33</sup>P, are in close proximity (FIG. 4) as determined by the mean pathlength for the  $\beta$ -particle in solution (Table 1).

Table 1

Radio-nuclide	Half-life	Mean $\beta$ energy (keV)	$\mu\text{m}$ path in H <sub>2</sub> O	Max. spec. Activity (Ci/mmol)
<sup>3</sup> H	12 yr	6	0.5	29
<sup>14</sup> C	5730 yr	50	43	0.062
<sup>125</sup> I	60 d	35	30	2180
<sup>35</sup> S	87 d	50	40	1500
<sup>33</sup> P	25 d	249	46	3000
<sup>32</sup> P	14 d	700	2170	9000

10 In the presence of bound radioactivity, the  $\beta$ -particle energy excites the scintillant to emit a visible photon. Unbound radioactivity, however, is too distant from the scintillant (and thus quenched by the solvent medium) to cause scintillation. The photon flux produced from the scintillant is proportional to the degree of phosphorylation. Unlike the scintillant proximity assay, the need to coat the plate with scintillation cocktail in the form of  
 15 fluoromicrospheres or beads is not necessary, since the hydrophobic anchor is also an effective fluorophore and scintillant. In the FLASHPLATE assay, 96-well microplates are chemically modified with a thin layer of polystyrene-based scintillant to provide a platform for nonseparation assays using a variety of isotopes without the addition of liquid scintillation cocktail. The SCINTI-PIPS assay greatly increases the efficiency by reducing the distance  
 20 between scintillant and  $\beta$ -emitter by several orders of magnitude.

Fluorophores consisting of bis-phenyl-substituted oxadiazoles have proven to be superior scintillants compared to those commonly used, such as 2,5-diphenyloxazole (PPO) and 1,4-bis([5-phenyl]-1,3-oxazol-2-yl)-benzene (POPOP). Independent studies have confirmed that the 2-(4-*t*-butyl-phenyl-5-biphenyl)-1,3,4-oxadiazole (FIG. 5) is approximately ten times more efficient than PPO in enhancing fluorography in SDS-PAGE gels, C.V. Sack, M.Sc. Thesis, State University of New York-Stony Brook (1988), reducing the amount of scintillant necessary to observe the same level of signal. Comparing the absorption and emission spectra of 2,5-diphenyl-1,3,4-oxadiazole (PPD) to that of PPO, the absorption maximum shifts from 310 nm for PPO to 280 nm for PPD (FIG. 5). Subsequently, the  
 25 emission maximum shifts from 370 nm for PPO to 350 nm for PPD. These hypochromic  
 30 emission properties are characteristic of the bis-phenyl-substituted oxadiazoles.

shifts are advantageous in detecting weak beta particles and because X-ray film used is most sensitive in the blue region centered around 350 nm. The number of nitrogen atoms in the five-membered ring of a conjugated system causes a hypochromic or blue shift in both the absorption and emission spectra. This type of fluorophore strongly absorbs in an effectively irreversible fashion to polystyrene surfaces as well as to other plastics. A PEG linker extends into the aqueous medium. G.M. Bonora, 117 Gazz. Chim. Ital. 379-380 (1987). PEG is used because of its biocompatibility, lack of toxicity and immunogenicity, and nonbiodegradability. S. Zalipsky, 6 Bioconjugate Chem. 150-165 (1995). Attachment of biologically relevant molecules (such as *sn*-1-*O*-(6-aminohexanoyl)PIP<sub>n</sub>s) to PEG is achieved either with commercially available bisfunctionalized PEG derivatives (Shearwater Polymers, Inc.) or with chemically desymmetrized PEG derivatives. A modular orientation such as this provides groups for specific coupling as well as creating an immobilized backbone that can be generalized to any receptor-ligand interaction.

#### Synthesis of SCINTI-PIPS

Two sets of building blocks are required: (1) the *sn*-1-*O*-(6-aminohexanoyl)PIP<sub>n</sub>s, and (2) the tetherable fluorophores. The PIP<sub>n</sub>s comprise eight molecular scaffolds based on the number and position of phosphates on the inositol head group (FIG. 6). PIP<sub>n</sub>s are commercially available (Echelon Research Laboratories, Salt Lake City, Utah), and synthetic routes are known. G.D. Prestwich et al., in Advances in Phosphoinositides (K. Bruzik & C.S. Chen eds; American Chemical Soc. 1998); Q.-M. Gu & G.D. Prestwich, 61 J. Org. Chem. 8642-8647 (1996); V.A. Estevez & G.D. Prestwich, 113 J. Am. Chem. Soc. 9885-9887 (1991); G. Dorman et al., 36 Tetrahedron Lett. 8719-8722 (1995); J. Chen et al., 61 J. Org. Chem. 6305-6312 (1996); O. Thum et al., 37 Tetrahedron Lett. 9017-9020 (1996).

Various acyl chains as well as the acyl-functionalized "tetherable" PIP<sub>n</sub>s have been prepared via such methods. A sample synthesis of acyl "tetherable" PI(4,5)P<sub>2</sub> is illustrated in FIG. 7. J. Chen et al., 61 J. Org. Chem. 6305-6312 (1996). Similar approaches have been developed for PI(3)P, PI(4)P, L. Feng et al., J. Org. Chem. (1998), and PI(5)P, J. Peng & G.D. Prestwich, Tetrahedron Lett. (1998). An alternative synthetic route involves the triester-modified PIP<sub>n</sub> derivatives. Q.-M. Gu & G.D. Prestwich, 61 J. Org. Chem. 8642-8647 (1996). Importantly, the chain-shortened analog di-C<sub>8</sub> PI(4,5)P<sub>2</sub>, and triester-modified BZDC-

PI(4,5)P<sub>2</sub> (FIG. 8) were both accepted as substrates for the PI3K. Analogous results have been obtained with PI(4)P 5-kinase and PI(5)P 4-kinases using triester-modified PIP<sub>n</sub> and chain-shortened analogs. These data demonstrate that acyl modifications are well-tolerated by PI kinases.

5 These PIP<sub>n</sub>s are immobilized using a strategy involving the tetherable scintillant fluorophore 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole ("amino-PBD"). The synthesis of amino-PBD is shown in FIG. 9. C.V. Sack, M.Sc. Thesis, State University of New York-Stony Brook (1988). This scintillant hydrophobically adsorbs to a plastic surface. A hydrophilic linker is used with or without a PEG spacer for coupling to a "tetherable" PIP<sub>n</sub>  
10 to form the immobilized phosphoinositide, SCINTI-PIPS, as shown in FIG. 3. Three tetherable SCINTI-LINKER modules are shown in FIG. 10.

15 The presence of the PEG linker addresses potential problems of water solubility and steric access of the kinase to the head group. As shown in FIG. 10, two illustrative embodiments of the invention use PEG-6 and PEG-85 linkers, i.e., where n=5 and n=85, respectively, whereas another illustrative embodiment uses a succinimide linker without a PEG linker ("PEG-0"). A recently-described, convenient route to desymmetrized PEGs may also be used. A. Schwabacher et al., 63 J. Org. Chem. 1727-1729 (1998). This method comprises preparing the symmetrical diazide followed by a biphasic mono-reduction method in which the amino-azide is continuously extracted into a reductant-free aqueous phase.

20 The SCINTI-LINKER modules comprise a short-chain hexa(ethyleneglycol) and the commercially available PEG 3,400. The relatively less expensive scintillants are chemically activated to provide a maximally economical convergent synthesis, as shown in FIGS. 10 and 11. Thus, the amino-PBD is succinylated and activated to an NHS ester for connection to the amino terminus of a desymmetrized PEG. Next, as for SCINTI-PEG0, the SCINTI-PEG6  
25 and SCINTI-PEG85 are further converted to active NHS esters for optimal coupling to the aminoacyl-PIP<sub>n</sub> as the limiting and more costly reagent.

#### Use of SCINTI-PIPS

30 SCINTI-PIPS plates are prepared by coating standard 96-well plates with several concentrations of each of four SCINTI-PIPS (FIG. 12) to determine which provides the most efficient substrate for kinase activity. These details are presented below. Importantly, the

present approach is benchmarked by two comparisons: (a) the FLASHPLATE type of SPA, and (b) coating with common non-covalent scintillators, e.g. PPO/POPOP, and commercial *t*-Bu-PBD are performed as described below (FIG. 12).

In the FLASHPLATE and non-covalent assays (FIG. 12, top and middle), the desired 5 di-C<sub>16</sub> PIP<sub>n</sub> (e.g. PI (Echelon Research Laboratories Cat. No. P-0016), PI(4)PP (Echelon Research Laboratories Cat. No. P-4016), PI(5)P (Echelon Research Laboratories Cat. No. P-5016) or PI(4,5)P2 (Echelon Research Laboratories Cat. No. P4516)) are coated onto a polystyrene microplate as unilamellar phospholipid vesicles (LUVs) formed by the extrusion method from a standard mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 10 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (PS) containing variable amounts (0.1 to 5%) of PIP<sub>n</sub>. M. Glaser et al., 271 J. Biol. Chem. 26187-26193 (1996). This mixed phospholipid coating (PE/PC in 2:1 ratio, or in a 1:2 ratio balanced with 10% PS) provides a balanced charged surface similar to that of a biological bilayer. For the non-covalent assay (FIG. 12, middle), 96-well 15 polystyrene assay plates are precoated with a scintillant fluorophore, e.g. a PPO/POPOP mixture or *t*-Bu-PBD, prior to application of the selected di-C<sub>16</sub> PIP<sub>n</sub> as a vesicle preparation. In the SCINTI-PIPS assay (FIG. 12, bottom), the PEG linker provides water solubility for the SCINTI-PIPS while preserving the hydrophobic adsorption by the aromatic scintillant. The PEG6 may provide the best balance of water solubility and adsorptive tenacity, but more 20 hydrophilic and more hydrophobic materials can be made since solubilities of the PIP<sub>n</sub>s change with increasing phosphorylation. Vesicle-type coating is merely illustrative, since other coating methods may be used. With the PEG0, for example, coating could proceed by evaporation of organic solvents, and with PEG85 a water solution could be used and evaporated under reduced pressure to allow adhesion of the SCINTI-PIP. It has been shown 25 that methanol, DMSO, and glacial acetic acid are compatible with the 96-well microtiter plate and may be used as coating solvents. Chloroform or any chloroform-methanol mixture is unsuitable.

In most lipid kinase assays, carrier lipids are required. For instance, PI3K will not phosphorylate PI(4)P or PI(4,5)P<sub>2</sub> in the absence of PS carrier, although it will phosphorylate 30 PI. The details for using an insect-cell expressed recombinant PI3K (as a glutathione (GST)-fusion protein) to form lipids is described below.

The PI3K assay is conducted as follows. The supernate from one cell pellet is diluted in NP-40 lysis buffer, snap frozen in liquid nitrogen, and stored as aliquots at -70°C. Under these conditions, the enzyme is stable for months. Crude enzyme from a 10,000 x g supernate can be used for PI3K assays. Alternatively, pure enzyme can be obtained from an 5 aliquot by thawing on ice, addition to a 1:1 slurry of GSH-beads (Pharmacia) pre-equilibrated in NP-40 lysis buffer, and incubation for 1 hr at 4°C. The beads are washed three times, once with PBS + 1% NP-40, then with 100 mM Tris-HCl pH 7.0 + 1 M LiCl, and finally with TNE (20 mM Tris-HCl pH 7 + 100 mM NaCl + 1 mM EDTA). The enzyme is eluted with 20 mM HEPES pH 7.0 + 1 mM EDTA containing 10 mM GSH, and aliquots of the PI3K preparation 10 are added to the 96-well plate. The reaction is initiated by addition of an aliquot (from 1-100 nCi of  $\gamma$ -<sup>33</sup>P-ATP) with a multichannel pipettor, allowed to proceed for 15 minutes at room temperature, and quenched by addition of 1 N HCl. After aspiration of the reaction medium liquid and washing with 100  $\mu$ l of reaction buffer, the plate is read in a 96-well plate reader, such as a Wallac MICROBETA unit or a Packard TOP COUNT unit. A typical screening 15 experiment contains (a) a control well (absence of  $\gamma$ -<sup>33</sup>P-ATP and inhibitor but coated with PS carrier), (b) a well with enzyme,  $\gamma$ -<sup>33</sup>P-ATP, and inhibitor, (c) a well with enzyme and  $\gamma$ -<sup>33</sup>P-ATP, and (d) a well with  $\gamma$ -<sup>33</sup>P-ATP and four times the normal enzyme concentration. Loading of the SCINTI-PIP on the plate with the enzyme and  $\gamma$ -<sup>33</sup>P-ATP concentrations can 20 be optimized such that good kinetics can be obtained under conditions in which the phosphoinositide substrate is in excess. Once this is achieved, the assay can be used to determine an effective IC<sub>50</sub> for the known PI3K inhibitors, wortmannin (irreversible) and LY294002 (reversible), as well as for screening and assaying unknown inhibitors.

In addition to recombinant PI3K, a complete panel of Type II PI3K isoforms has been obtained by Dr. Andrew J. Morris (State University of New York-Stony Brook), including 25 p85 $\alpha$ , P110 $\alpha$ , p110 $\beta$ , and p110 $\gamma$ , in which an epitope tag on the p85 $\alpha$  facilitates rapid purification of the catalytically active heterodimeric complex.

Unlike the SPA or FLASHPLATE assays, the need to coat the microplate with a scintillant is unnecessary with SCINTI-PIPS, since the tetherable hydrophobic anchor is simultaneously a highly efficient adhesive moiety as well as scintillant. Thus, the proximity 30 between the scintillant and  $\beta$ -emitter is intramolecular (within 40 to 100 Å) and several orders of magnitude greater than the micrometer proximity achieved with the coating protocols,

greatly increasing efficiency. These assays can be conducted in 96-well polystyrene microtiter plates with the medium-energy isotope  $^{33}\text{P}$ . Use of  $^{32}\text{P}$  would not be appropriate, since the higher energy of this isotope would result in adjacent wells responding to radioisotope in a different well.

5 Once the substrate (in this case, PI(4,5)P<sub>2</sub>) is immobilized, a phosphorylation reaction takes place in each well in the presence of a cellular extract, reaction buffer, and enzyme (e.g. insect cell expressed PI3K). The reaction is initiated upon the addition of  $\gamma$ - $^{33}\text{P}$ -ATP. If bound (within a 10  $\mu\text{m}$  distance of scintillant), the  $\beta$ -particle is brought in close enough proximity that it stimulates the scintillant to emit light. However, energy from unbound 10 radioactivity is quenched by solvent (FIG. 4). To give a lower background, a simple wash step can be included after quenching to ensure removal of excess unbound  $^{33}\text{P}$ , while plate-bound  $^{33}\text{P}$  incorporated into the immobilized phosphoinositide substrate can be assessed by liquid scintillation counting. M. Susa et al., 267 J. Biol. Chem. 22951-22956 (1992); R.H. Palmer et al., 270 J. Biol. Chem. 22412-22416 (1995); C.L. Carpenter et al., 268 J. Biol. 15 Chem. 9478-9483 (1993).

Table 2 shows illustrative substrates, products, and PI kinases that can be assayed according to the present invention.

Table 2

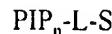
Substrate	Product with PI3K	Product with PI4K	Product with PI5K
PI	PI(3)P	PI(4)P	PI(5)P
PI(3)P	--	PI(3,4)P <sub>2</sub>	PI(3,5)P <sub>2</sub>
PI(4)P	PI(3,4)P <sub>2</sub>	--	PI(4,5)P <sub>2</sub>
PI(5)P	PI(3,5)P <sub>2</sub>	--	--
PI(3,4)P <sub>2</sub>	--	--	PI(3,4,5)P <sub>3</sub>
PI(4,5)P <sub>2</sub>	PI(3,4,5)P <sub>3</sub>	--	--
PI(3,5)P <sub>2</sub>	--	PI(3,4,5)P <sub>3</sub>	--

30 The publications and other reference materials referred to herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference.

## CLAIMS

I claim:

1. A composition represented by the formula



5 wherein  $\text{PIP}_n$  is a phosphoinositide polyphosphate, L in a linker moiety, and S is a scintillant.

2. The composition of claim 1 wherein  $\text{PIP}_n$  is a member selected from the group consisting of phosphatidylinositol, phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol 5-phosphate, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 3,4-bisphosphate, phosphatidylinositol 3,5-bisphosphate, and 10 phosphatidylinositol 3,4,5-trisphosphate.

3. The composition of claim 2 wherein  $\text{PIP}_n$  is phosphatidylinositol.

4. The composition of claim 2 wherein  $\text{PIP}_n$  is phosphatidylinositol 3-phosphate.

5. The composition of claim 2 wherein  $\text{PIP}_n$  is phosphatidylinositol 4-phosphate.

6. The composition of claim 2 wherein  $\text{PIP}_n$  is phosphatidylinositol 5-phosphate.

15. 7. The composition of claim 2 wherein  $\text{PIP}_n$  is phosphatidylinositol 4,5-bisphosphate.

8. The composition of claim 2 wherein  $\text{PIP}_n$  is phosphatidylinositol 3,4-bisphosphate.

9. The composition of claim 2 wherein  $\text{PIP}_n$  is phosphatidylinositol 3,5-bisphosphate.

20 10. The composition of claim 2 wherein  $\text{PIP}_n$  is phosphatidylinositol 3,4,5-trisphosphate.

11. The composition of claim 1 wherein L is a hydrophilic linker moiety.

25 12. The composition of claim 11 wherein L is a member selected from the group consisting of succinimide and poly(ethylene glycol) linkers.

13. The composition of claim 12 wherein L is a succinimide linker.

14. The composition of claim 12 wherein L is a polyethylene glycol linker.

15. The composition of claim 14 wherein said poly(ethylene glycol) linker comprises hexa(ethylene glycol).

30 16. The composition of claim 14 wherein said poly(ethylene glycol) linker comprises PEG 3,400.

17. The composition of claim 2 wherein L is a member selected from the group consisting of succinimide and poly(ethylene glycol) linkers.

18. The composition of claim 17 wherein L is a succinimide linker.

19. The composition of claim 17 wherein L is a poly(ethylene glycol) linker.

5 20. The composition of claim 19 wherein said poly(ethylene glycol) linker comprises hexa(ethylene glycol).

21. The composition of claim 19 wherein said poly(ethylene glycol) linker comprises PEG 3,400.

10 22. The composition of claim 1 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

23. The composition of claim 2 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

24. The composition of claim 12 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

15 25. The composition of claim 17 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

26. A composition represented by the formula

$\text{PIP}_n\text{-L-S-Matrix}$

20 wherein  $\text{PIP}_n$  is a phosphoinositide polyphosphate, L is a linker moiety, S is a scintillant, and Matrix is a solid support.

27. The composition of claim 26 wherein  $\text{PIP}_n$  is a member selected from the group consisting of phosphatidylinositol, phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol 5-phosphate, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 3,4-bisphosphate, phosphatidylinositol 3,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate.

28. The composition of claim 27 wherein  $\text{PIP}_n$  is phosphatidylinositol.

29. The composition of claim 27 wherein  $\text{PIP}_n$  is phosphatidylinositol 3-phosphate.

30. The composition of claim 27 wherein  $\text{PIP}_n$  is phosphatidylinositol 4-phosphate.

31. The composition of claim 27 wherein  $\text{PIP}_n$  is phosphatidylinositol 5-

phosphate.

32. The composition of claim 27 wherein PIP<sub>n</sub> is phosphatidylinositol 4,5-bisphosphate.

5 33. The composition of claim 27 wherein PIP<sub>n</sub> is phosphatidylinositol 3,4-bisphosphate.

34. The composition of claim 27 wherein PIP<sub>n</sub> is phosphatidylinositol 3,5-bisphosphate.

10 35. The composition of claim 27 wherein PIP<sub>n</sub> is phosphatidylinositol 3,4,5-trisphosphate.

36. The composition of claim 26 wherein L is a hydrophilic linker moiety.

37. The composition of claim 36 wherein L is a member selected from the group consisting of succinimide and poly(ethylene glycol) linkers.

15 38. The composition of claim 37 wherein L is a succinimide linker.

39. The composition of claim 37 wherein L is a polyethylene glycol linker.

40. The composition of claim 39 wherein L comprises hexa(ethylene glycol).

41. The composition of claim 39 wherein L comprises PEG 3,400.

42. The composition of claim 27 wherein L is a member selected from the group consisting of succinimide and poly(ethylene glycol) linkers.

15 43. The composition of claim 42 wherein L is a succinimide linker.

44. The composition of claim 42 wherein L is a polyethylene glycol linker.

45. The composition of claim 44 wherein L comprises hexa(ethylene glycol).

46. The composition of claim 44 wherein L comprises PEG 3,400.

47. The composition of claim 26 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

25 48. The composition of claim 27 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

49. The composition of claim 37 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

30 50. The composition of claim 42 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

51. The composition of claim 26 wherein said Matrix is comprised of a

hydrophobic polymer.

52. The composition of claim 51 wherein said hydrophobic polymer comprises polystyrene.

53. The composition of claim 51 wherein said hydrophobic polymer is in the form of a microtitre plate.

54. The composition of claim 27 wherein said Matrix is comprised of a hydrophobic polymer.

55. The composition of claim 54 wherein said hydrophobic polymer comprises polystyrene.

10 56. The composition of claim 54 wherein said hydrophobic polymer is in the form of a microtitre plate.

57. The composition of claim 37 wherein said Matrix is comprised of a hydrophobic polymer.

15 58. The composition of claim 57 wherein said hydrophobic polymer comprises polystyrene.

59. The composition of claim 57 wherein said hydrophobic polymer is in the form of a microtitre plate.

60. The composition of claim 42 wherein said Matrix is comprised of a hydrophobic polymer.

20 61. The composition of claim 60 wherein said hydrophobic polymer comprises polystyrene.

62. The composition of claim 60 wherein said hydrophobic polymer is in the form of a microtitre plate.

25 63. The composition of claim 47 wherein said Matrix is comprised of a hydrophobic polymer.

64. The composition of claim 63 wherein said hydrophobic polymer comprises polystyrene.

65. The composition of claim 63 wherein said hydrophobic polymer is in the form of a microtitre plate.

30 66. A method for assaying a phosphatidylinositol kinase comprising:  
(a) providing a composition represented by the formula

PIP<sub>n</sub>-L-S-Matrix

wherein PIP<sub>n</sub> is a phosphoinositide polyphosphate, L is a linker moiety, S is a scintillant, and Matrix is a solid support;

- (b) contacting the composition with effective amounts of a sample to be tested  
5 containing such phosphatidylinositol kinase, reaction buffer, and ATP labeled with a low-energy  $\beta$ -emitter to form a reaction mixture;
- (c) incubating the reaction mixture for a sufficient time and under suitable  
10 conditions for the phosphatidylinositol kinase to phosphorylate the PIP<sub>n</sub> such that the low-energy  $\beta$ -emitter is coupled thereto, whereupon radioactive decay of the low-energy  $\beta$ -emitter induces emission of light by the scintillant; and
- (d) detecting the light.

67. The method of claim 66 wherein said low-energy  $\beta$ -emitter is  $^{33}\text{P}$ .

68. The method of claim 66 wherein PIP<sub>n</sub> is a member selected from the group consisting of phosphatidylinositol, phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol 5-phosphate, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 3,4-bisphosphate, phosphatidylinositol 3,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate.

69. The method of claim 66 wherein L is a hydrophilic linker moiety.

70. The method of claim 69 wherein L is a member selected from the group  
20 consisting of succinimide and poly(ethylene glycol) linkers.

71. The method of claim 66 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

72. The method of claim 66 wherein said Matrix is comprised of a hydrophobic polymer.

25 73. The method of claim 72 wherein said hydrophobic polymer comprises polystyrene.

74. The method of claim 72 wherein said hydrophobic polymer is in the form of a microtitre plate.

75. A method of making a composition represented by the formula

PIP<sub>n</sub>-L-S

30 wherein PIP<sub>n</sub> is a phosphoinositide polyphosphate, L is a linker moiety, and S is a scintillant

comprising:

(a) activating the scintillant to result in an activated scintillant, and reacting the activated scintillant with the linker moiety, thereby obtaining a linker-scintillant intermediate;

5 (b) activating the linker-scintillant to result in an activated linker-scintillant intermediate, and reacting the activated linker-scintillant intermediate with an aminoacyl-PIP<sub>n</sub>, thereby obtaining the composition represented by the formula PIP<sub>n</sub>-L-S.

10 76. The method of claim 75 wherein said PIP<sub>n</sub> is a member selected from the group consisting of phosphatidylinositol, phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol 5-phosphate, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 3,4-bisphosphate, phosphatidylinositol 3,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate.

15 77. The method of claim 75 wherein L is a hydrophilic linker moiety.

78. The method of claim 77 wherein L is a member selected from the group consisting of succinimide and poly(ethylene glycol) linkers.

15 79. The method of claim 75 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

80. The method of claim 75 wherein said aminoacyl-PIP<sub>n</sub> is *sn*-1-*O*-(6-aminohexanoyl), 2-*O*-hexanoyl-PIP<sub>n</sub>.

20 81. A method of making a composition represented by the formula  
PIP<sub>n</sub>-L-S-Matrix

wherein PIP<sub>n</sub> is a phosphoinositide polyphosphate, L is a linker moiety, S is a scintillant, and Matrix is a solid support comprising adsorbing PIP<sub>n</sub>-L-S to the Matrix.

25 82. The method of claim 81 wherein said PIP<sub>n</sub> is a member selected from the group consisting of phosphatidylinositol, phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol 5-phosphate, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 3,4-bisphosphate, phosphatidylinositol 3,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate.

83. The method of claim 81 wherein L is a hydrophilic linker moiety.

30 84. The method of claim 83 wherein L is a member selected from the group consisting of succinimide and poly(ethylene glycol) linkers.

85. The method of claim 81 wherein S is 2-(4-amino-methylphenyl)-5-(4-

biphenyl)-1,3,4-oxadiazole.

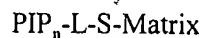
86. The method of claim 81 wherein said Matrix is comprised of a hydrophobic polymer.

87. The method of claim 86 wherein said hydrophobic polymer comprises polystyrene.

88. The method of claim 86 wherein said hydrophobic polymer is in the form of a microtitre plate.

89. A method for screening compounds for a drug that interferes with phosphatidylinositol kinase activity comprising:

10 (a) providing a composition represented by the formula



wherein  $\text{PIP}_n$  is a phosphoinositide polyphosphate, L in a linker moiety, S is a scintillant, and Matrix is a solid support;

15 (b) contacting the composition with effective amounts of a compound to be tested, reaction buffer, phosphatidylinositol kinase, and ATP labeled with a low-energy  $\beta$ -emitter to form a reaction mixture;

20 (c) incubating the reaction mixture for a sufficient time and under suitable conditions for the phosphatidylinositol kinase to phosphorylate the  $\text{PIP}_n$  such that the low-energy  $\beta$ -emitter is coupled thereto, whereupon radioactive decay of the low-energy  $\beta$ -emitter induces emission of light by the scintillant;

(d) measuring the light and comparing the amount of light measured to an amount of light emitted from a control reaction that lacks the compound, wherein a decrease of light in the presence of the compound indicates interference with the phosphatidylinositol kinase activity.

25 90. The method of claim 89 wherein said low-energy  $\beta$ -emitter is  $^{33}\text{P}$ .

91. The method of claim 89 wherein  $\text{PIP}_n$  is a member selected from the group consisting of phosphatidylinositol, phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol 5-phosphate, phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 3,4-bisphosphate, phosphatidylinositol 3,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate.

30 92. The method of claim 89 wherein L is a hydrophilic linker moiety.

93. The method of claim 89 wherein L is a member selected from the group consisting of succinimide and poly(ethylene glycol) linkers.

94. The method of claim 89 wherein S is 2-(4-amino-methylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.

5 95. The method of claim 89 wherein said Matrix is comprised of a hydrophobic polymer.

96. The method of claim 95 wherein said hydrophobic polymer comprises polystyrene.

10 97. The method of claim 95 wherein said hydrophobic polymer is in the form of a microtitre plate.

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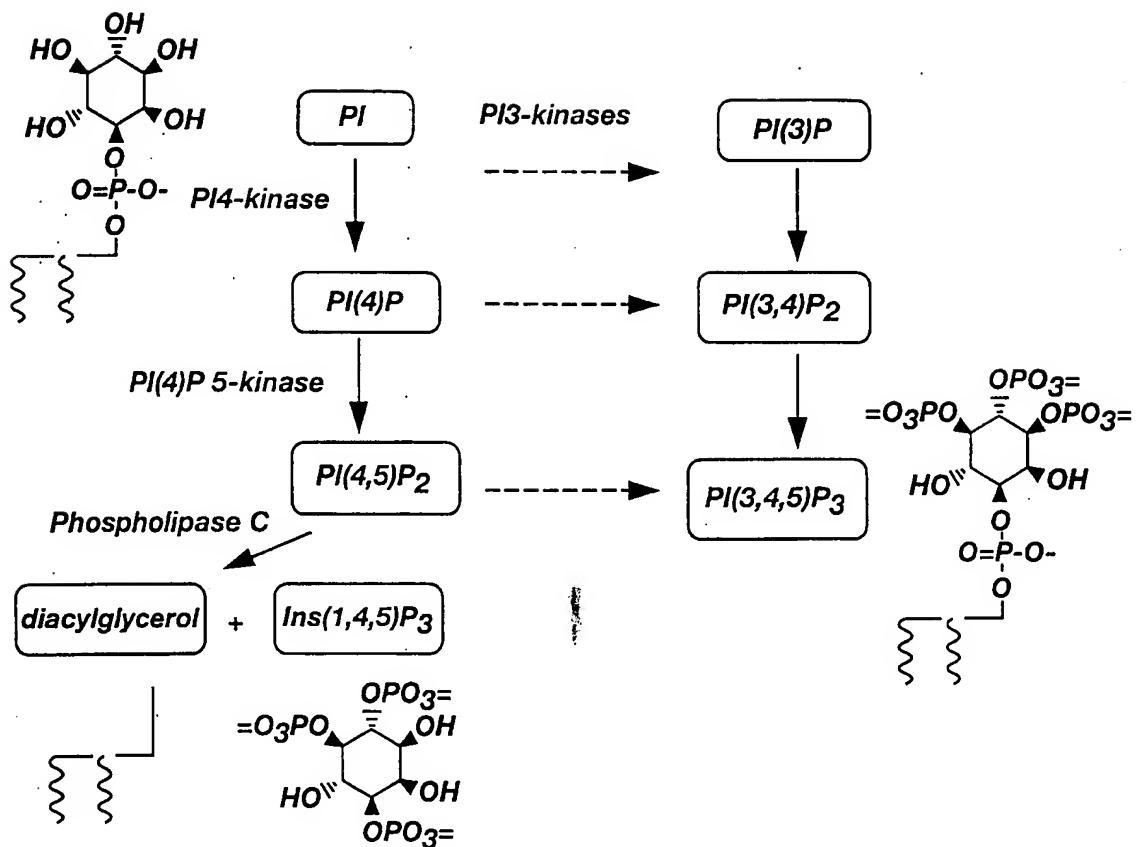


Fig. 1

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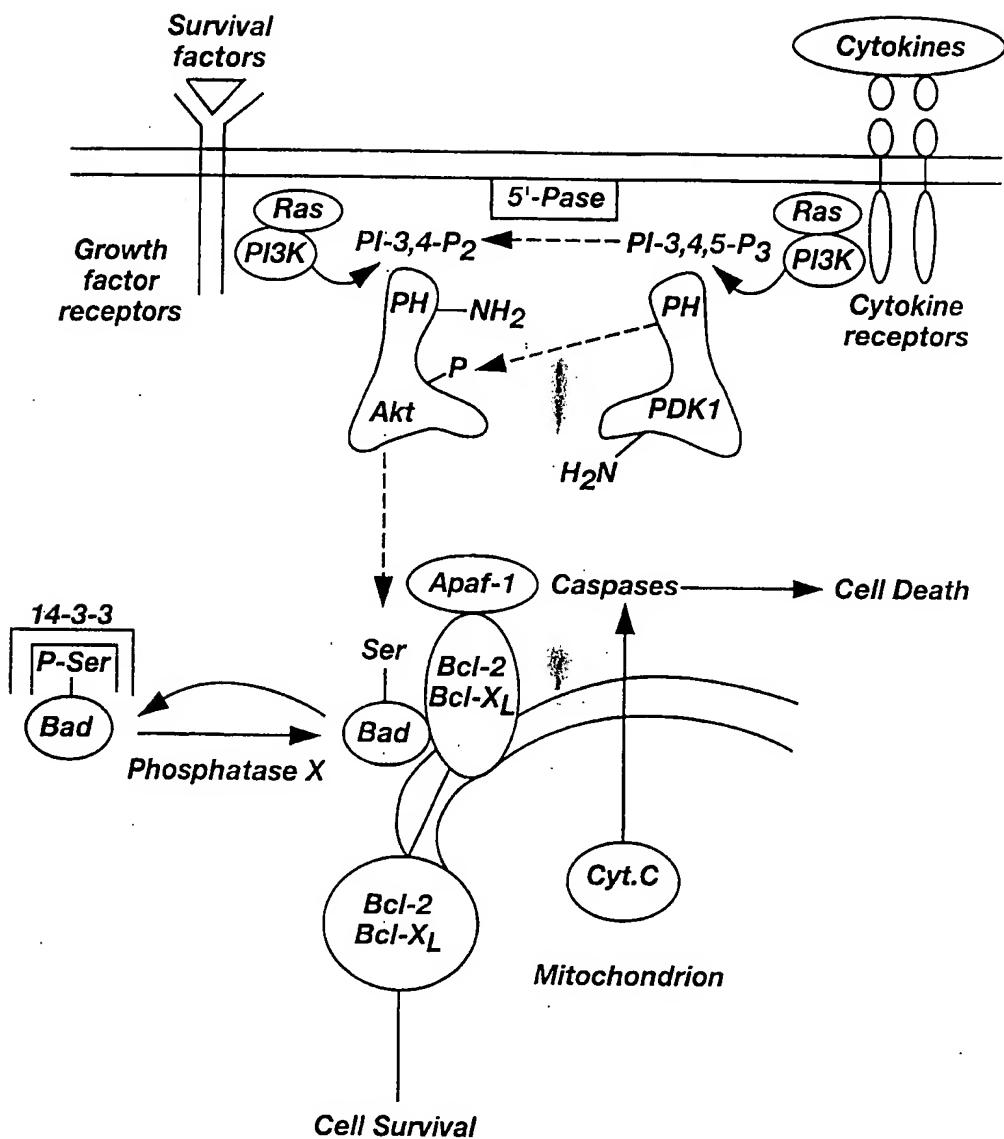


Fig. 2

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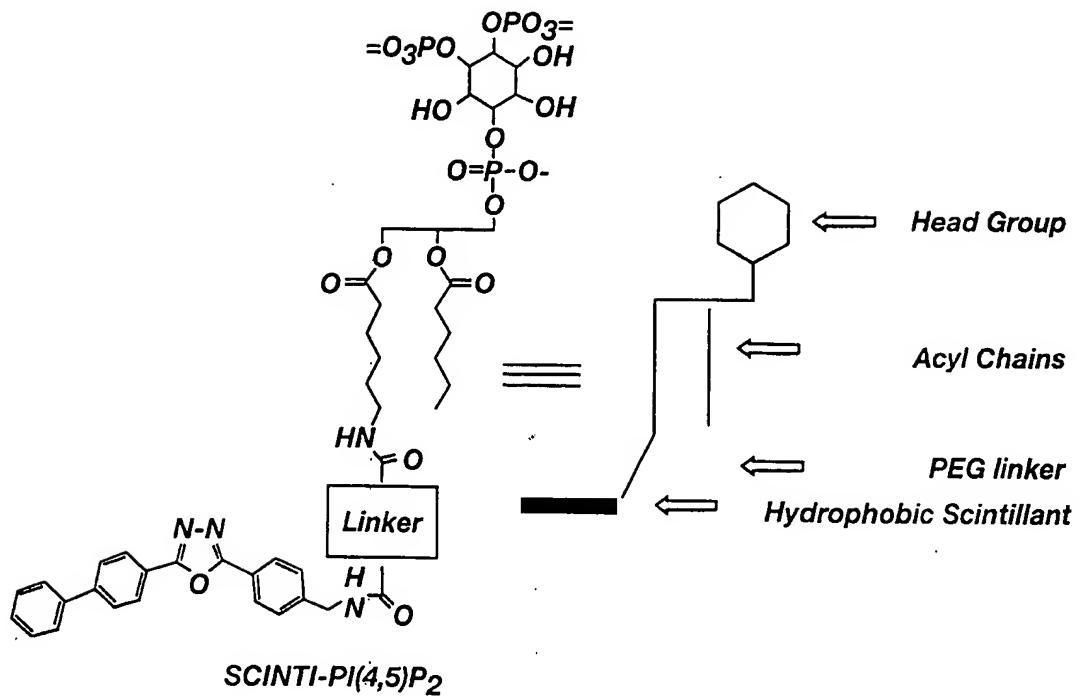


Fig. 3

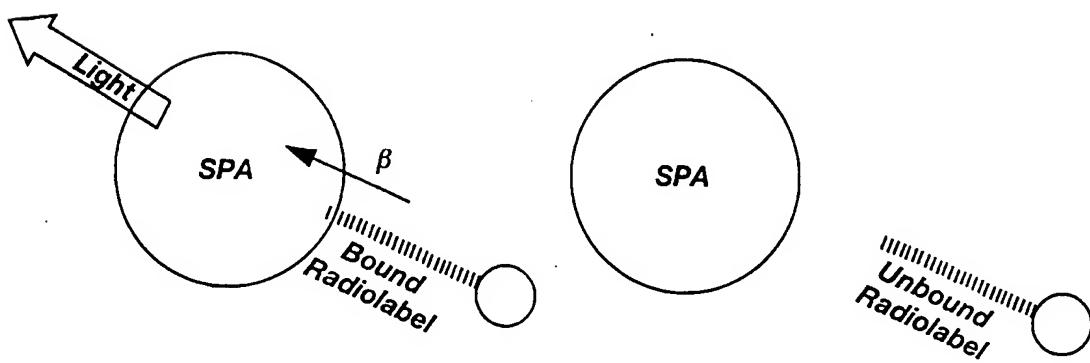
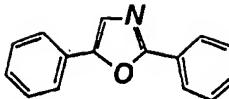
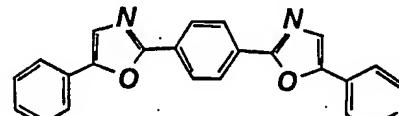
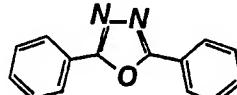
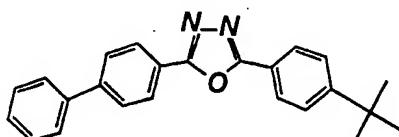
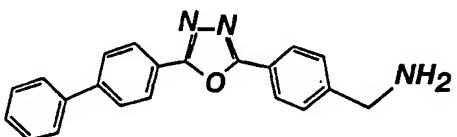


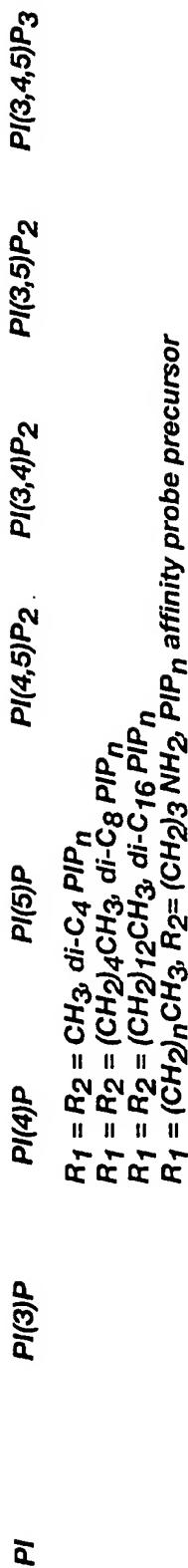
Fig. 4

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	$\lambda_{\text{absorption}}$	$\lambda_{\text{emission}}$
		
<b>2,5-diphenyl-1,3-oxazole (PPO)</b>	<b>310 nm</b>	<b>370 nm</b>
		
<b>1,4-bis([5-phenyl]-1,3-oxazol-2-yl)-benzene (POPOP)</b>	<b>360 nm</b>	<b>410 nm</b>
		
<b>2,5-diphenyl-1,3,4-oxadiazole (PPD)</b>	<b>280 nm</b>	<b>350 nm</b>
	<b>305 nm</b>	<b>365 nm</b>
<b>2-(4-t-butylphenyl)-5-biphenyl-1,3,4-oxadiazole (t-Bu-PBD)</b>		
	<b>(305) nm</b> <i>estimated values</i>	<b>(365) nm</b> <i>estimated values</i>
<b>2-(4-aminomethylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (Aminomethyl-PBD)</b>		

*Fig. 5*

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$R_1 = R_2 = CH_3, di-C_4 PIP_n$   
 $R_1 = R_2 = (CH_2)_4 CH_3, di-C_8 PIP_n$   
 $R_1 = R_2 = (CH_2)_{12} CH_3, di-C_{16} PIP_n$   
 $R_1 = (CH_2)_n CH_3, R_2 = (CH_2)_3 NH_2, PIP_n$  affinity probe precursor

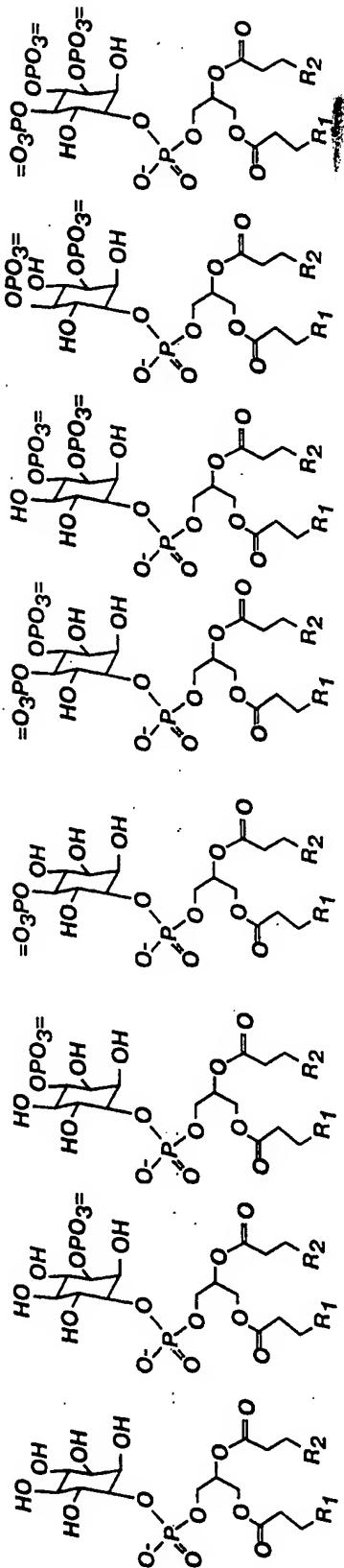


Fig. 6

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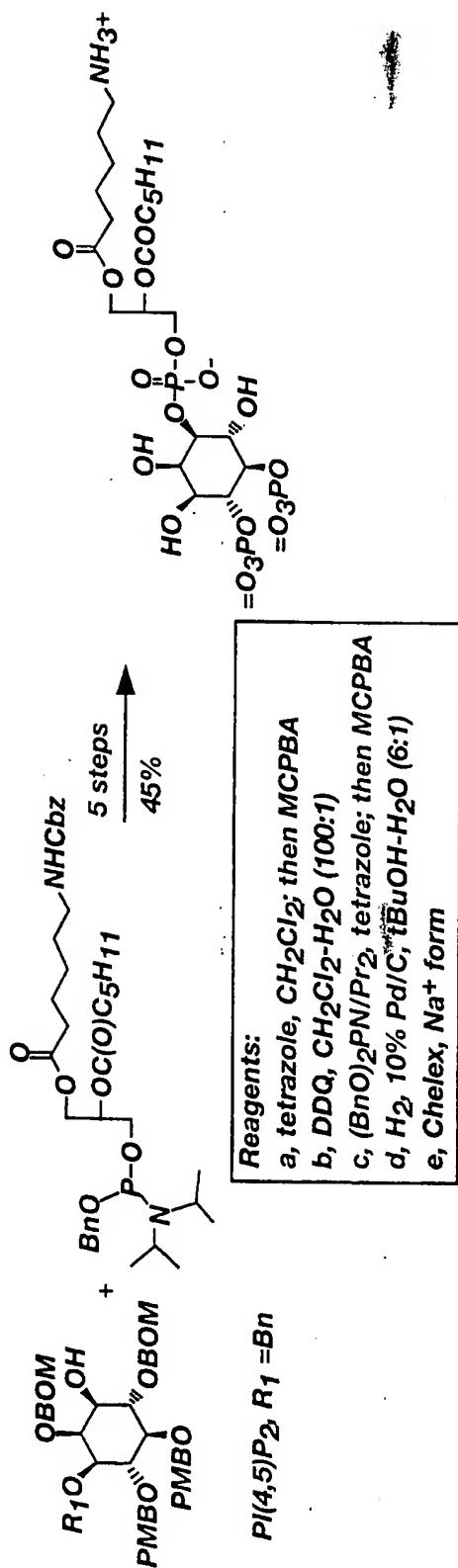


Fig. 7

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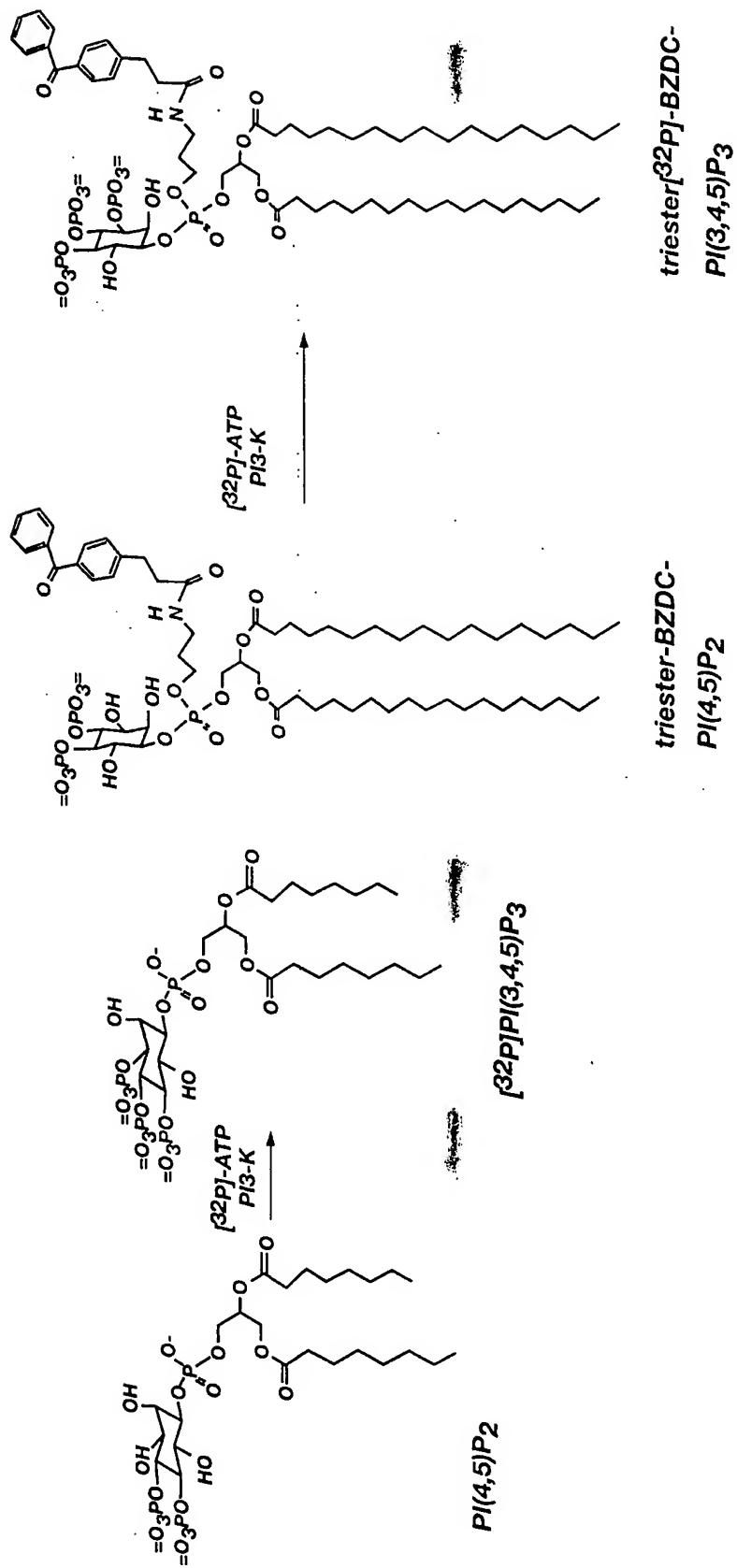


Fig. 8

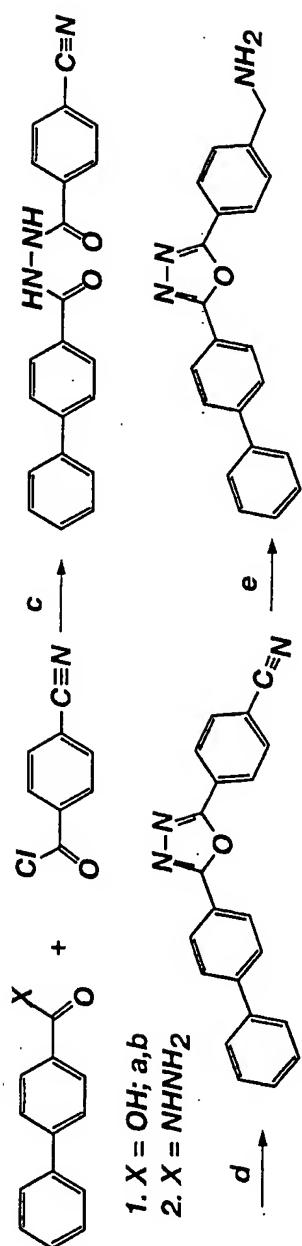
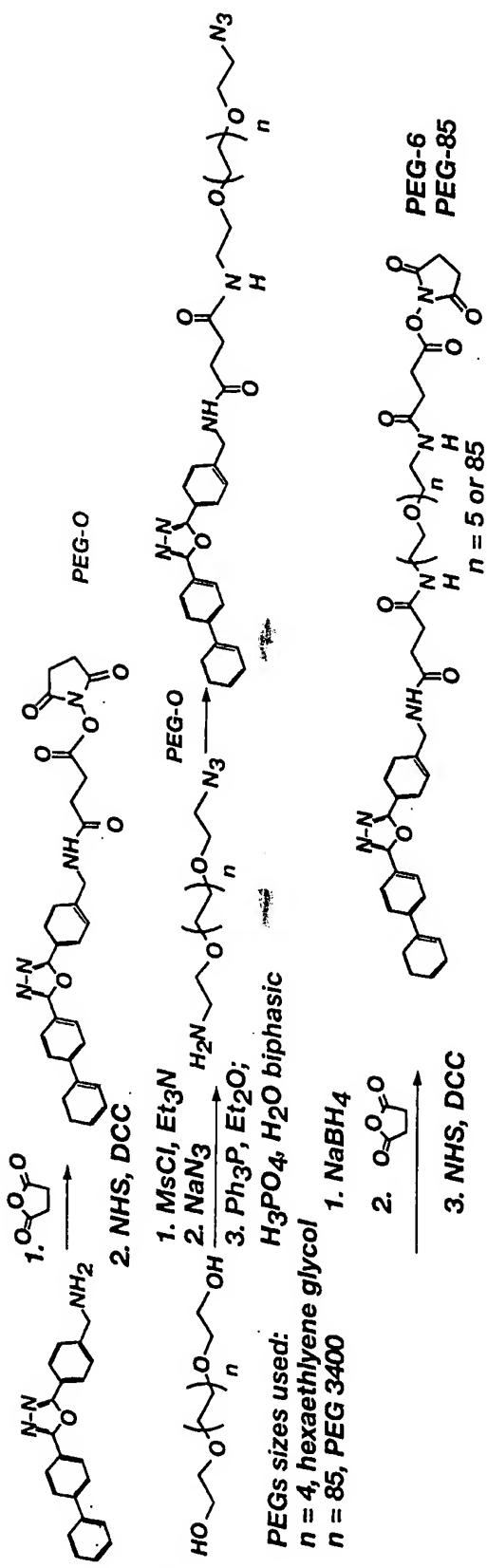


Fig. 9

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SUBSTITUTE SHEET (RULE 26)

Fig. 10

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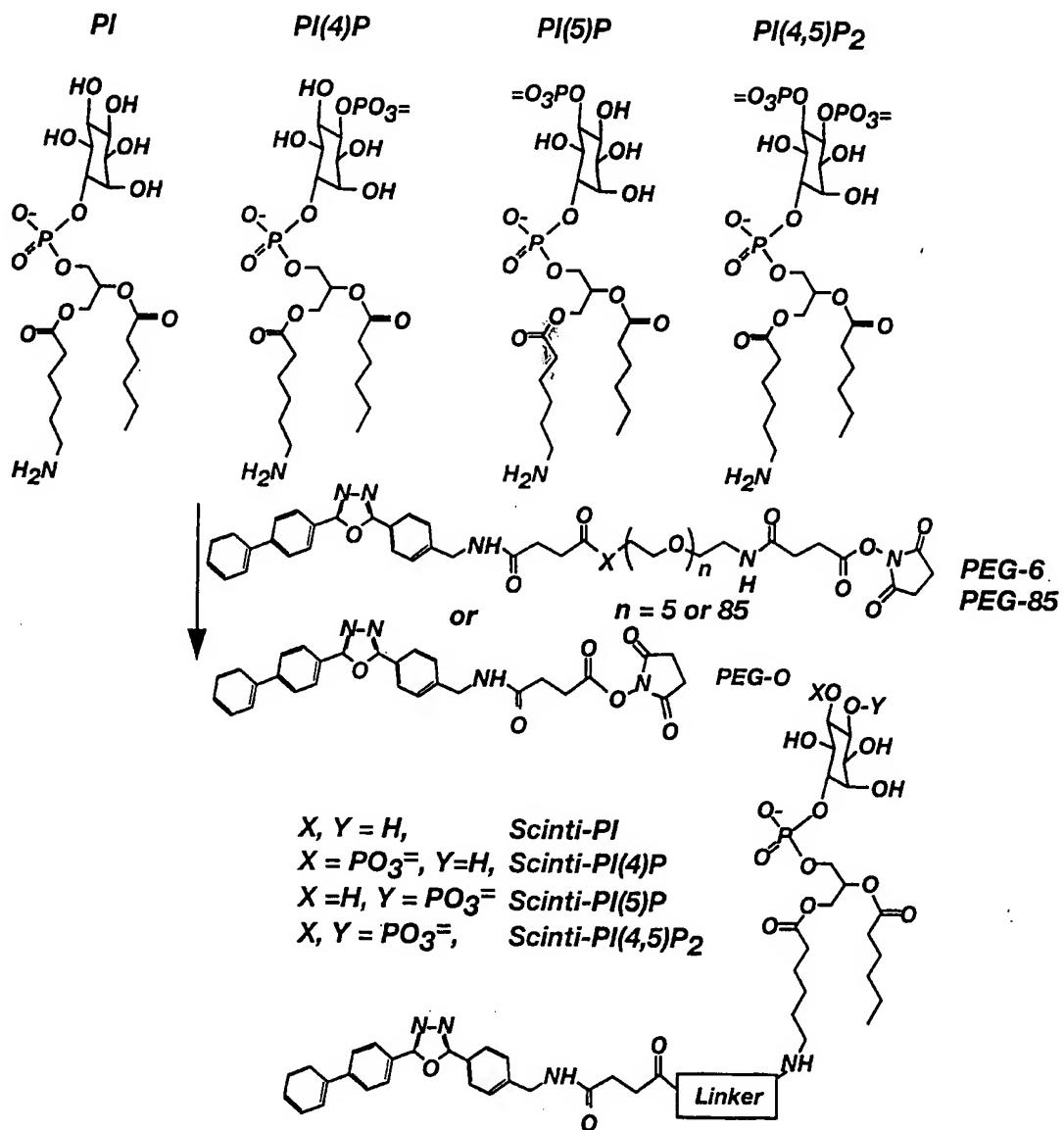


Fig. 11

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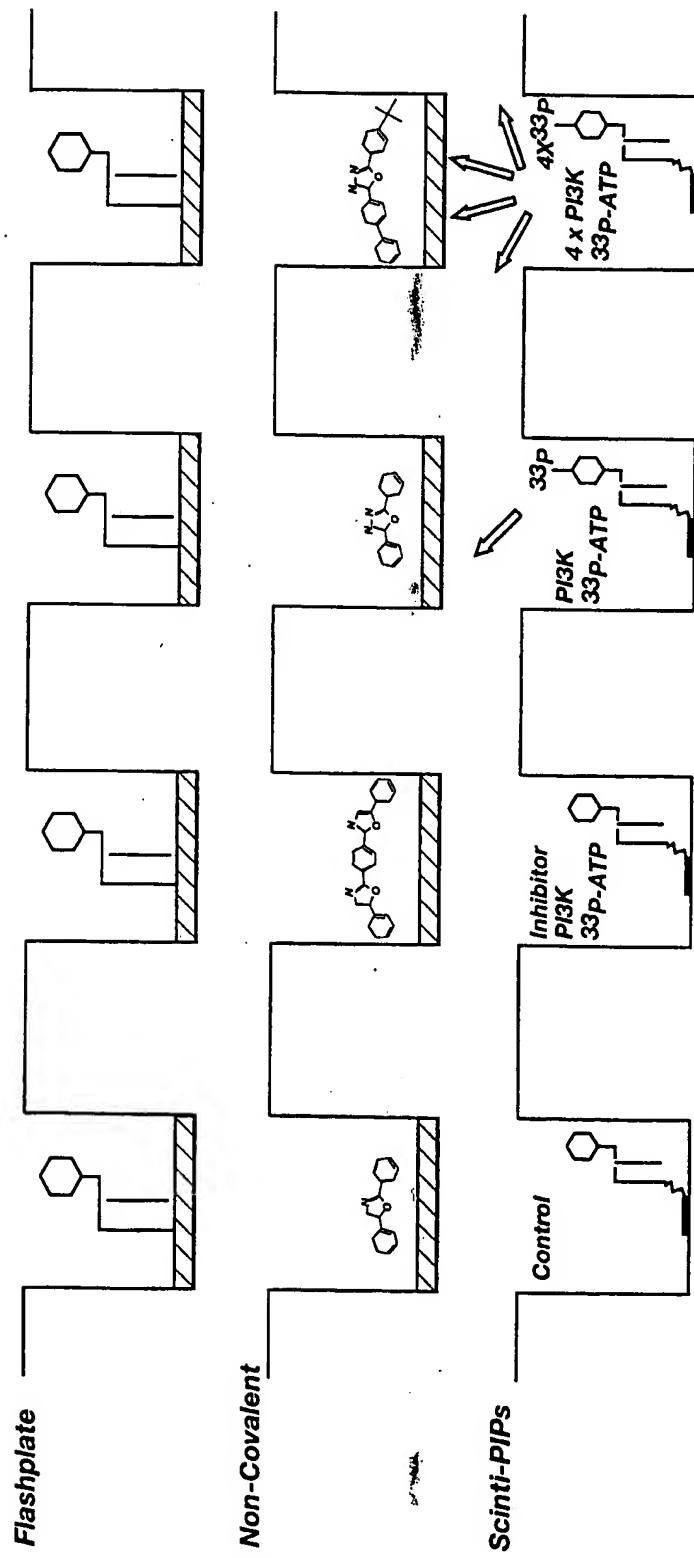


Fig. 12